Histochem Cell Biol (2001) 116:97–107
DOI 10.1007/s004180100296

ROBERT FEULGEN PRIZE LECTURE

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The secretory membrane system studied in real-time

Accepted: 11 June 2001 / Published online: 17 July 2001

Abstract The discovery and development of green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, has revolutionized studies on protein localization and dynamics by allowing direct observation of a protein's life history and pathway in living cells, previously only deduced from genetic, biochemical, or immunolabeling studies. Applied to the secretory membrane system, which regulates delivery of newly synthesized proteins and lipids to the cell surface, GFP-based studies are providing important new insights into the maintenance and biogenesis of organelles, as well as the origin, pathway, and fate of secretory transport intermediates.

Keywords Green fluorescent protein · Endoplasmic reticulum · Golgi apparatus · Pre-Golgi intermediates · Membrane traffic · Protein mobility · Membrane cycling

Introduction

With the advent of green fluorescent protein (GFP) technology (reviewed in Tsien 1998), the subcellular localization, dynamics, and pathways of a protein can be studied in living cells. Other applications in living cells include the visualization of RNA processing and transport (Dirks et al. 2001), the dynamics of nuclear proteins (Houtsmuller and Vermeulen 2001), the localization of GFP-open reading frame fusion proteins (Simpson et al. 2001), imaging of specific cell populations in complex organs (Christensen et al. 2001; Lundstrom et al. 2001; Tucker 2001; Wahlfors et al. 2001) and in targeted or transgenic mice (Hadjantonakis and Nagy 2001). Virtually any protein can be tagged with GFP, a beta-barrel-shaped protein that contains an amino acid triplet (ser-tyr-gly) in its center that cyclizes to become a fluorophore. The GFP fluorophore has a high fluorescence yield, which makes it bright, and it is resistant to photobleaching, the photo-induced alteration of a fluorophore that extinguishes its fluorescence. This allows proteins fused to GFP to be visualized easily within cells with low light illumination over long periods. Molecular engineering of the GFP coding sequence, furthermore, has allowed cell expression of GFP to be optimized, both at the level of protein expression and fluorophore optimization. Finally, the GFP tag usually does not affect parent protein function and becomes fluorescent within a short time after it is synthesized and folded within cells.

The study of secretory membrane trafficking is one area of research in which GFP fusion proteins are having a significant impact (Lippincott-Schwartz et al. 2001a). Eukaryotic cells contain an elaborate secretory membrane system that allows newly synthesized protein complexes to be efficiently inserted on their cell surface, secreted into the extracellular space, or targeted to other membrane-bound compartments within the cell (Fig. 1). This system is comprised of the endoplasmic reticulum (ER), in which luminal and transmembrane proteins are synthesized, the Golgi apparatus, in which secretory products are processed and sorted, and transport intermediates, which convey proteins and lipids between the ER and Golgi and from the Golgi to the plasma membrane. GFP fusion proteins used in a variety of fluorescent imaging techniques to study protein dynamics in this system have allowed researchers to follow for the first time the origin and fate of transport intermediates, and their path through the cytoplasm in living cells. They also have allowed analysis of the lateral mobility of proteins residing in different compartments, and the morphological transformations of organelles during mitosis or after treatment with membrane-trafficking perturbants. This review discusses the recent advances in GFP imaging and how they have broadened our understand-

Robert Feulgen Prize Lecture 2001 presented at the 43rd Symposium of the Society for Histochemistry in Vienna, Austria, on 28 September 2001

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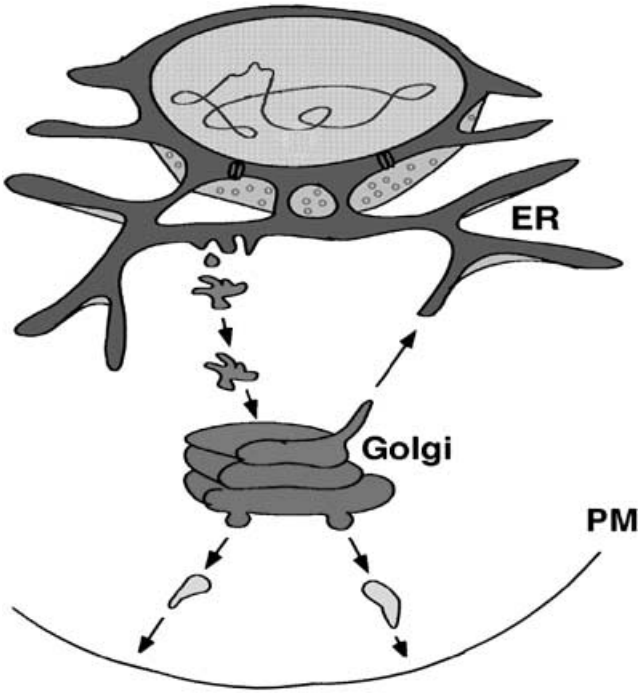

Fig. 1 Overview of the secretory pathway. Newly synthesized secretory cargo in the endoplasmic reticulum (*ER*) is packaged into transport intermediates that target to the Golgi apparatus. In the Golgi, cargo is processed and then conveyed by post-Golgi carriers to the plasma membrane (*PM*). Both pre- and post-Golgi intermediates normally use microtubules to track through the cytoplasm

ing of the regulation, maintenance, and biogenesis of the secretory membrane traffic.

GFP imaging techniques

Three types of GFP imaging techniques have been most useful for investigating the properties of the secretory membrane system: time-lapse imaging, dual-color labeling, and photobleaching. Time-lapse imaging of GFP-expressing cells involves the collection of multiple images over time. The brightness and photostability of GFP fluorescence combined with the sensitivity of modern imaging systems (for example, a wide-field fluorescence microscope incorporating a CCD camera or a laser scanning confocal microscope) make it very easy to perform this type of experiment with minimal photobleaching and photodamage. Experiments employing time-lapse imaging of GFP chimeras have investigated processes within the secretory pathway that are dynamic, including vesicle budding, transport, and fusion (Presley et al. 1997; Scales et al. 1997; Hirschberg et al. 1998; Nakata et al. 1998; Toomre et al. 1999), and organelle breakdown and reassembly (Storrie et al. 1998; Zaal et al. 1999; Lippincott-Schwartz and Zaal 2000).

The availability of GFP variants with differing excitation and emission spectra offer the possibility of double labeling in order to simultaneously visualize two proteins in a cell. The most widely used pair are yellow fluorescent protein and cyan fluorescent protein (Ellenberg et al. 1998; Keller et al. 2001). A different pair that is also available for dual-color labeling experiments are enhanced GFP, which is excited at 488 nm and emits at 510 nm, and red fluorescent protein (Matz et al. 1999), which excites at 558 nm and emits at 583 nm. The latter pair can be used with commonly used laser lines on a confocal microscope or with filter sets that are widely available on fluorescent wide-field microscopes. In addition to their usefulness in dual-color time-lapse imaging, the GFP variant pairs are being used in FRET experiments to detect direct interactions between proteins (Periasamy and Day 1999; Pollok and Heim 1999).

When exposed to high illumination levels, the GFP fluorophore can be photobleached and this is the basis for the third widely used GFP imaging technique. Photobleaching can be used to investigate the mobility of GFP-tagged proteins (Lippincott-Schwartz et al. 1999, 2001a, b; White and Stelzer 1999). The fluorescent molecules in a region of a cell are photobleached by intense illumination and the movement of non-bleached molecules into the bleached region of fluorescence from other areas of the cell is monitored by imaging at low-level illumination. The rate at which fluorescence recovers provides a measure of the mobility of the tagged protein. This technique is called fluorescence recovery after photobleaching or FRAP (Edidin 1994).

A different photobleaching technique called FLIP, for fluorescence loss in photobleaching, monitors loss of fluorescence rather than fluorescence recovery. Here, fluorescence in one area of a cell is repeatedly photobleached while images of the entire cell are collected. If fluorescent molecules from other regions of the cell can diffuse into the area being bleached, loss of fluorescence occurs from both places, indicating that the regions are connected and the protein can diffuse between them. Experiments employing FLIP have helped clarify the extent of continuity of various intracellular membrane compartments (Cole et al. 1996b; White et al. 1999; Zaal et al. 1999).

Another application of photobleaching is selective photobleaching to remove specific populations of GFP fluorescence within a cell. This technique can be used to enhance the imaging of dim structures within cells or in areas of the cell next to very bright objects. Alternatively, it can be used to create transients in a steady-state population. For example, by selectively photobleaching one of several compartments where a population of GFP molecules resides, it is possible to measure the rate of exchange of GFP molecules between the compartments. Selective photobleaching performed in this manner has been used to measure the rate of vesicular transport between compartments (Hirschberg et al. 1998; Zaal et al. 1999; Dahm et al. 2001), as well as the rate of exchange of cytosolic proteins on and off membranes (Vasudevan et al. 1998; Stephens et al. 2000).

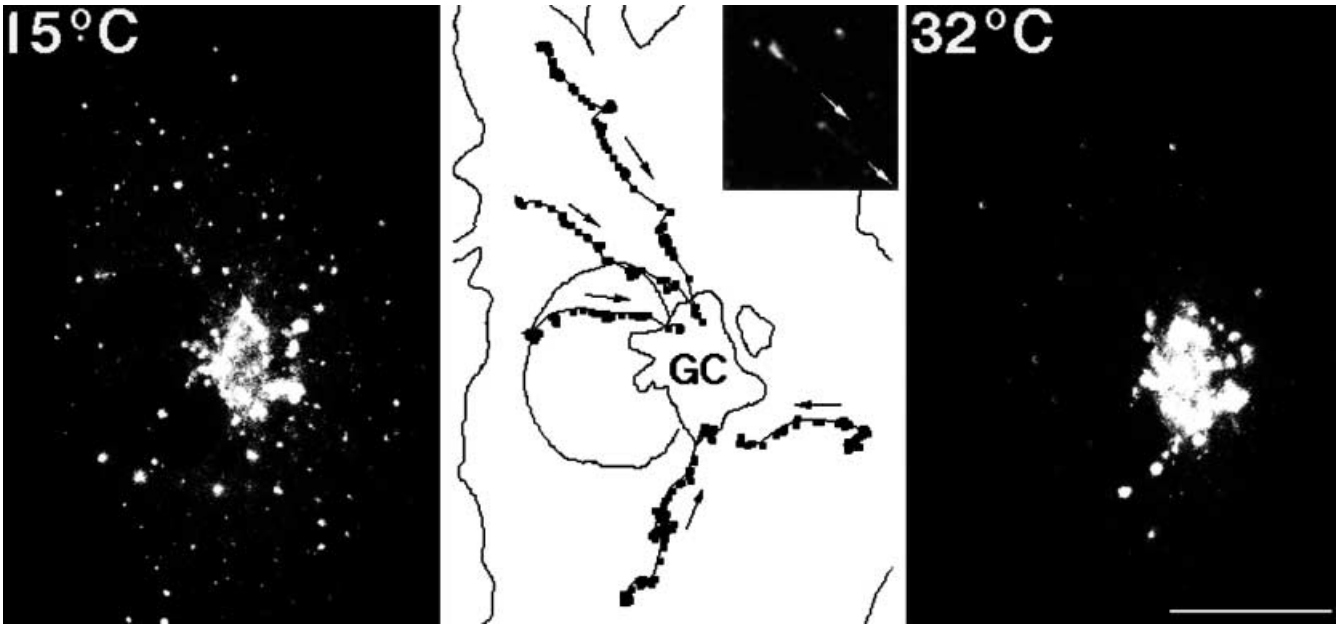

Fig. 2 Time-lapse imaging of ER-to-Golgi transport using vesicular stomatitis viral G protein tagged with green fluorescent protein (VSVG-GFP). Cells expressing VSVG-GFP were shifted from 40°C to 15°C for several hours to accumulate the fluorescent protein in pre-Golgi structures localized in peripheral sites throughout the cell. Upon warm-up to 32°C, the pre-Golgi structures can be seen tracking through the cytoplasm along curvilinear tracks (*arrows*) toward the Golgi (*GC*) where they dock and fuse. The data demonstrate that pre-Golgi structures have a transient existence within cells

Characteristics of secretory transport

The secretory pathway is comprised of distinct membrane-bounded compartments (i.e., ER, Golgi, and plasma membrane) and transport intermediates that as a system regulate the synthesis, processing, and plasma membrane delivery of newly synthesized protein and lipid within cells. In this system, secretory cargo is synthesized and assembled in the ER, and then delivered to the Golgi apparatus for remodeling by glycoprotein and glycolipid processing enzymes. Cargo is subsequently transported from the Golgi to the plasma membrane and to other final destinations within the cell. GFP-based, time-lapse imaging studies, which take advantage of GFP's brightness and resistance to photobleaching, have permitted analysis of the origin, pathway, and fate of secretory transport intermediates.

ER-to-Golgi transport

Delivery of proteins from the ER to the Golgi apparatus involves discrete membrane-bound structures, also called pre-Golgi intermediates, vesicular tubular clusters, or ER-to-Golgi intermediate compartment (ERGIC) (Saraste and Kuismanen 1992; Bannykh et al. 1996; Hauri et al. 2000), that accumulate newly synthesized secretory cargo prior to its delivery to the Golgi apparatus. The properties of these structures, including enrichment in particular proteins and a distinct morphology (i.e., elaborate tubular clusters adjacent to ER exit sites), led to the original hypothesis that they were a stable compartment between the ER and Golgi apparatus capable of budding anterograde (ER-to-Golgi) or retrograde (Golgi-to-ER) vesicles (Lotti et al. 1992; Tang et al. 1995; Klumperman et al. 1998).

Studies visualizing ER-to-Golgi traffic in living cells using the secretory cargo protein ts045 vesicular stomatitis viral G protein tagged with GFP (VSVG-GFP) support a different view of the role of pre-Golgi intermediates in ER-to-Golgi trafficking. The ts045 VSVG protein misfolds and is retained in the ER at 40°C and upon shift to 32°C moves as a synchronous population to the Golgi apparatus before being transported to the plasma membrane (Bergmann 1989). These properties of VSVG are preserved upon addition of GFP to the cytoplasmic tail of the protein (Presley et al. 1997; Scales et al. 1997), making it an ideal reporter for analyzing secretory trafficking within living cells. In time-lapse experiments, VSVG-GFP was found to concentrate within seconds of temperature shift into bright, fluorescent pre-Golgi structures that were widely distributed at ER sites scattered throughout the cytoplasm (Presley et al. 1997; Scales et al. 1997; Shima et al. 1999). After filling up with VSVG-GFP, the pre-Golgi structures moved as single units away from ER exit sites, stretching into long tubular elements as they translocated unidirectionally through the cytoplasm (Fig. 2). No loss of fluorescent material upon transport was observed. Upon reaching the Golgi apparatus, the intermediates fused en bloc with it. These results are contrary to the prediction that pre-Golgi structures represent a stable compartment. Instead, they support the view that pre-Golgi structures are dynamic vehicles for ER-to-Golgi traffic, which exist only transiently within cells.

Time-lapse imaging experiments using VSVG-GFP further showed that the movement and fate of pre-Golgi intermediates depends on microtubules. These cytoplasmic filaments emanate from the centrosomal region of cells and form a polarized radial array, with microtubule minus ends converging at the cell center and plus ends scattered in the cell periphery. In cells treated with nocodazole to depolymerize microtubules, VSVG-GFP is efficiently exported out of the ER into peripheral pre-Golgi elements, but these structures remain at peripheral sites over time, never fusing with Golgi membranes at the microtubule-organizing center (Presley et al. 1997). A similar phenotype is observed when the microtubule motor complex of dynein/dynactin is disrupted, suggesting dynein/dynactin powers the translocation of pre-Golgi structures along microtubules (Presley et al. 1997). Interestingly, over long periods of time in nocodazole, pre-Golgi structures at ER exit sites grow large and eventually differentiate into Golgi stacks (Cole et al. 1996a; Storrie et al. 1998). This suggests that in addition to being a vehicle for cargo transport and sorting, pre-Golgi intermediates can serve as precursors of new Golgi elements when they are unable to translocate to and fuse with pre-existing Golgi structures.

Pre-Golgi intermediates are known to sort and recycle selected components back to the ER (Tang et al. 1995; Klumperman et al. 1998) and this property has been visualized in time-lapse imaging studies using SNARE (soluble NSF attachment protein receptor) proteins, including rsec22b and rbet1 (Chao et al. 1999) and p58 (Roberts et al. 1999), the rat homologue of ERGIC53 (Lahtinen et al. 1992; Hauri et al. 2000), a lectin-like protein thought to have a role in forward transport of specific cargo out of the ER (Appenzeller et al. 1999; Moussalli et al. 1999) expressed as GFP chimeras in cells. The chimeras were found to localize to ER exit sites and did not move with pre-Golgi intermediates toward the Golgi apparatus. Whereas the GFP-tagged SNARE proteins remained in close proximity to ER exit sites, consistent with rapid cycling back to the ER, p58-GFP segregated out from the pre-Golgi intermediates later as tubule processes that moved to the cell periphery.

Golgi-to-plasma membrane transport

The mechanisms and carriers involved in protein delivery from the Golgi to the plasma membrane are also being clarified using GFP chimera in time-lapse imaging experiments. After delivery to the Golgi apparatus, VSVG-GFP segregated into discrete domains within Golgi membranes (Keller and Simons 1997; Hirschberg et al. 1998; Toomre et al. 1999). These domains then elongated into tubules before detaching from the Golgi body as post-Golgi carriers (Hirschberg et al. 1998; Toomre et al. 1999). Active membrane sorting appears to be involved in the formation in these carriers. Not only are Golgi resident proteins, such as furin, excluded from the VSVG-GFP enriched tubules (Hirschberg et al. 1998), but different cargo proteins are capable of being sorted into different post-Golgi carriers. In dual-color, time-lapse imaging experiments with cyan and yellow spectral variants of GFP attached to basolateral cargo (VSVG) or apical cargo (glycosyl phosphatidylinositol; GPI), respectively, distinct post-Golgi carriers enriched in either VSVG or GPI were observed (Keller et al. 2001).

Severing of membrane tubules enriched in secretory cargo extending out from Golgi membranes has been shown using GFP live cell imaging techniques to be mediated by dynamin-2, a Golgi-localized GTPase and mechanoenzyme (Jones et al. 1998). In cells cotransfected with a dominant-negative dynamin-2 and GFP-tagged cargo, the delivery of cargo to the plasma membrane was impeded and cargo accumulated in anastomosing tubular arrays extending out from the Golgi complex (Cao et al. 2000; Kreitzer et al. 2000). An actin-based cytoskeletal system also could help to sever Golgi-derived tubules involved in plasma membrane transport (Müsch et al. 1997) given that cytochalasin B treatment (which disrupts the actin cytoskeleton) slows down traffic from Golgi to the plasma membrane and causes Golgi-derived tubules containing VSVG-GFP to become longer and to detach more slowly from the Golgi (Hirschberg et al. 1998).

After detaching as tubules from the Golgi complex, post-Golgi carriers carrying GFP-containing cargo have been shown to undergo dramatic shape changes, including extension, retraction, and bifurcation (Hirschberg et al. 1998; Nakata et al. 1998; Toomre et al. 1999; Polishchuk et al. 2000; Fig. 3). The carriers can extend as tubules up to 3–5 μm into the cell periphery and have been shown to be capable of carrying up to 10,000 VSVG-GFP molecules (Hirschberg et al. 1998). Correlative light-electron microscopy, a technique where structures observed in vivo are then fixed and examined by electron microscopy, has revealed that the post-Golgi carriers can be as large as half the size of Golgi cisternae and that they can exist as tubular-saccular structures (Nakata et al. 1998; Polishchuk et al. 2000). Movement of post-Golgi carriers, which occurs at rates up to 2.7 μm/s, requires microtubules (Hirschberg et al. 1998; Nakata et al. 1998; Toomre et al. 1999) and the activity of the plus-end directed motor protein kinesin (Kreitzer et al. 2000). During translocation to the cell periphery, the post-Golgi carriers do not intersect with other pathways or membranes within the cell (Toomre et al. 1999; Polishchuk et al. 2000). Upon reaching the edge of the cell, the carriers remain stationary for a variable period (15–30 s), and then rapidly fuse and disperse their contents in the plasma membrane. A detailed analysis of fusion of post-Golgi carriers with the plasma membrane has been recently studied at the level of single carriers using total internal reflection microscopy (Schmoranzer et al. 2000; Toomre et al. 2000).

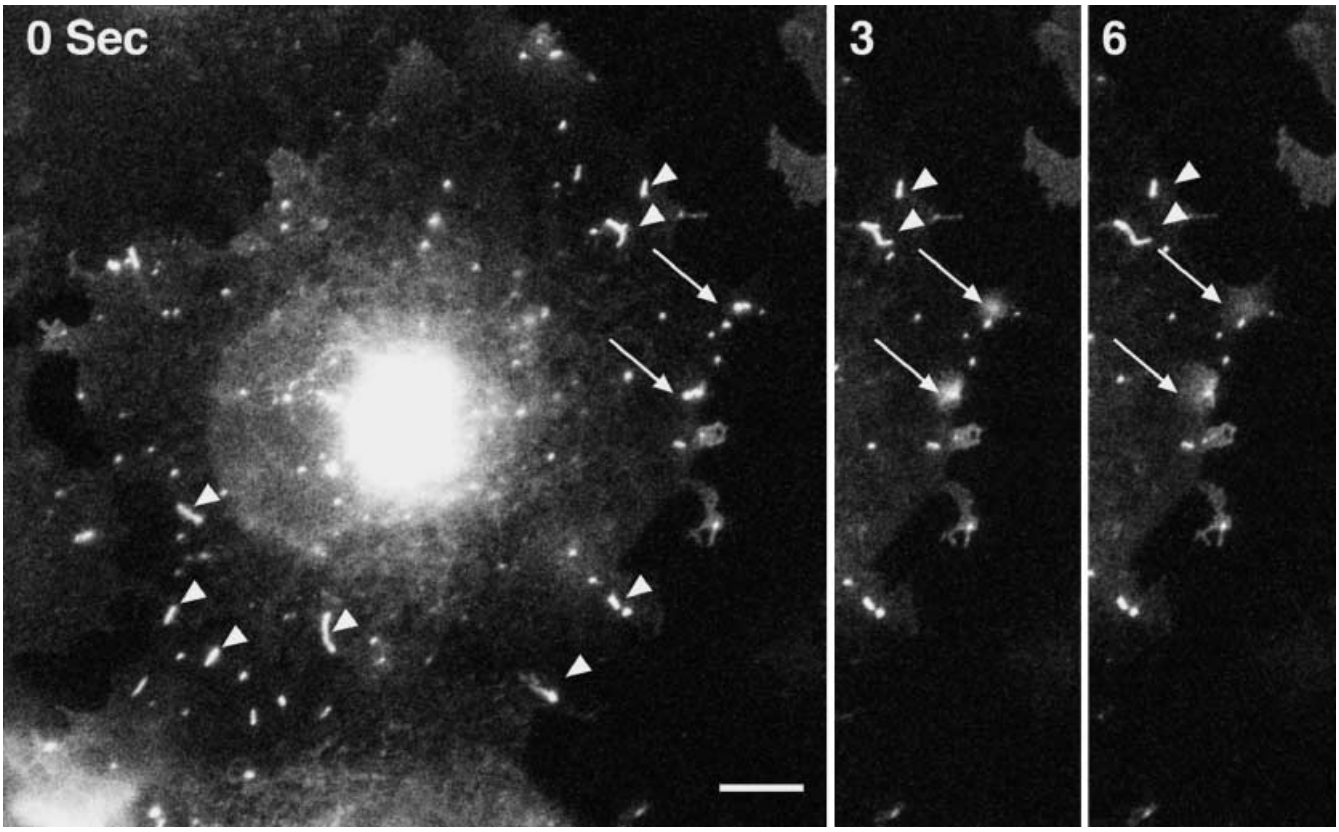

Fig. 3 Dynamics of post-Golgi carriers containing VSVG-GFP. Approximately 40 min after shifting to permissive temperature in cells expressing VSVG-GFP, the fluorescent protein has reached the Golgi apparatus and is being actively packaged into post-Golgi carriers. These large transport intermediates undergo striking shape changes (*arrowheads*) during transport and fuse directly with the plasma membrane (*arrows*) without intersecting other membrane transport pathways

Secretory trafficking kinetics

The kinetic properties of secretory transport, including how long cargo resides in a particular compartment and the rate of cargo influx into and efflux out of a given compartment, has been addressed by quantitative time-lapse imaging of single cells expressing GFP-tagged cargo molecules (Hirschberg et al. 1998). In these experiments, cells are imaged under conditions where all intracellular fluorescent molecules are detectable at any one time. This usually requires the use of a 25× objective with NA of 0.8, which allows the entire cell to be maintained in the center of the focal depth during imaging. Hirschberg et al. (1998) performed such an analysis to describe VSVG trafficking upon shift from 40°C to 32°C. Changes in fluorescence intensity of VSVG-GFP in the Golgi region, and in the entire cell, were measured and plotted as VSVG-GFP moved through the secretory pathway. The changes were then used to address whether a kinetic model with specific rate parameters could fit the data. A simple model consisting of a series of linear rate laws connecting three compartments (ER, Golgi, and plasma membrane) arranged in series was able to fit the data. This indicates that the secretory pathway can be viewed as a series of first-order reactions (ER-to-Golgi, Golgi-to-plasma membrane, and plasma membrane-to-degradative site), each governed by a single rate-limiting step. Moreover, because the rate constants describing these steps did not change as the concentrations of VSVG-GFP in different compartments went from high (early in the experiment) to low (late in the experiment), the data imply that transport machinery can accommodate large changes in cargo load. For ER-to-Golgi transport the mean rate constant was 2.8% per min, for Golgi-to-plasma membrane transport it was 3.0% per min, and for transport from the plasma membrane to a degradative site it was 0.25% per min. Interestingly, VSVG-GFP molecules were found arriving at the plasma membrane within 10 min after shift to permissive temperature, which suggests that there are no lags for VSVG-GFP transport through the Golgi complex. The use of GFP imaging to analyze secretory kinetics has only just begun, but provides a powerful approach for studying the most fundamental parameters of protein trafficking, including protein transport rates and residency times under different conditions and in different cell types. These parameters of protein trafficking have been only crudely estimated, if at all, in traditional biochemical pulse-chase studies.

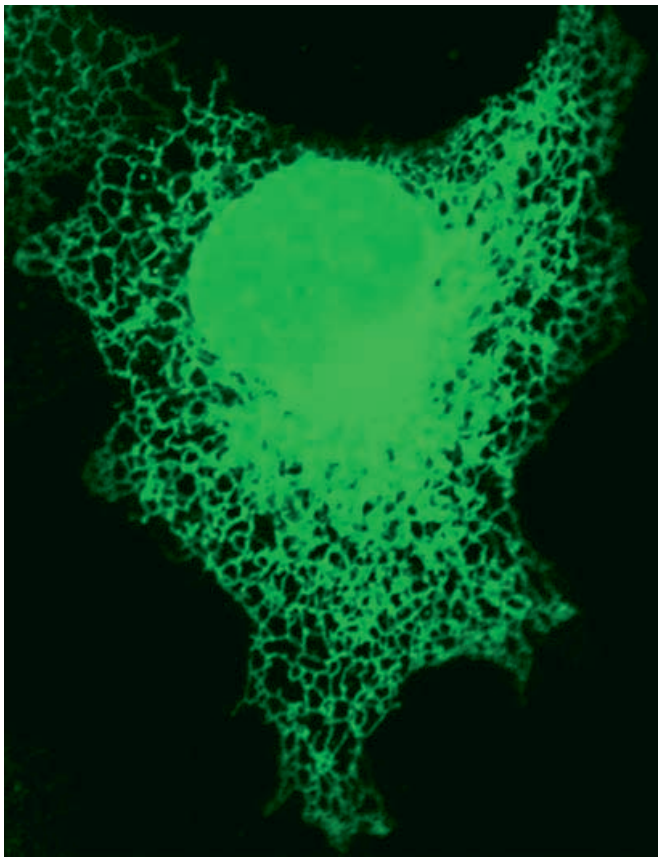

Fig. 4 Morphology of the ER shown in a COS cell expressing VSVG-GFP at 40°C

Organelle dynamics

Protein mobility and retention in the ER

The ER is comprised of an extensive array of interconnecting membrane tubules and cisternae that extend throughout the cell, including the nuclear envelope (Fig. 4). Photobleaching experiments have been used to determine whether ER membranes comprise a single, continuous system or are organized into stable subcompartments (for example, rough versus smooth domains). In these experiments, GFP-tagged luminal and membrane ER markers were found to rapidly recover into a photobleach box with no regions restricted for diffusional exchange of these molecules (Cole et al. 1996b; Subramanian and Meyer 1997; Dayel et al. 1999; Nehls et al. 2000). Moreover, when a small area of ER membranes expressing the chimeras was repeatedly bleached in FLIP experiments, complete loss of ER-associated fluorescence from the photobleached cell was observed (including the nuclear envelope; Cole et al. 1996b; Dayel et al. 1999). These results indicate that the membranes and luminal spaces of the ER are normally continuous throughout the cell.

Quantitative FRAP experiments have revealed that the diffusion coefficient, *D*, of many GFP-tagged ER membrane proteins ranges between 0.2 and 0.5 $\mu m^2/s$ (Cole et al. 1996b; Ellenberg et al. 1997; Nehls et al. 2000). Since this is near the theoretical limit for protein diffusion in a bilayer (Hughes et al. 1982), the data suggest that the proteins are not impeded in their lateral mobility. The high mobility of many ER proteins could be important for ensuring the diverse processes occurring within this compartment (Hammond and Helenius 1995), which include protein synthesis, folding, processing, and assembly. Interestingly, FRAP experiments comparing *D* for misfolded and correctly folded ts045 VSVG-GFP localized to the ER revealed that both proteins diffused near the theoretical limit for a protein in a bilayer (Nehls et al. 2000). This indicates that misfolded VSVG complexes are not retained in the ER as a result of forming an immobilized aggregate or being tethered to a meshwork. Retention of VSVG and potentially other misfolded proteins in the ER, therefore, could be due to their failure to be actively sorted to ER exit sites.

The ability to probe the diffusional characteristics of GFP-tagged proteins in the ER by photobleaching is providing insight into the physical-chemical properties of the ER. For example, diffusion of soluble GFP within the ER lumen is three- to sixfold slower than GFP in the cytoplasm (Dayel et al. 1999), which indicates that the ER lumen is more viscous than cytoplasm. Interestingly, ATP depletion (which crosslinks the chaperone BiP to substrates) decreased the mobility of soluble GFP in the ER lumen, while tunicamycin treatment (which prevents addition of carbohydrate onto glycoproteins) increased its mobility (Nehls et al. 2000). These results imply that protein folding machinery and branched carbohydrate side chains on glycoproteins play important roles in determining ER luminal viscosity. Diffusion of soluble GFP in the ER lumen is also inhibited under conditions where the ER is vesiculated or fragmented. These changes in ER architecture have been shown to occur when cytosolic calcium levels within cells are greatly increased (Terasaki et al. 1996; Subramanian and Meyer 1997).

Golgi morphology and dynamics

The Golgi apparatus receives newly synthesized proteins and lipids from the ER, covalently modifies them, and then packages them for delivery to the plasma membrane, lysosomes, secretory granules, or back to the ER for reutilization. How the Golgi's unique structure, comprised of polarized stacks of cisternae and surrounding tubules and vesicles, subserves its diverse roles in secretory trafficking is not understood (Lippincott-Schwartz et al. 1998; Lippincott-Schwartz and Zaal 2000). Studies using GFP fusion proteins have analyzed the dynamics of protein movement associated with the Golgi in different cell types. In so doing, they are providing insight into the mechanisms underlying Golgi biogenesis and maintenance.

GFP-tagged Golgi resident proteins expressed in plant cells have revealed an intimate positioning of ER and

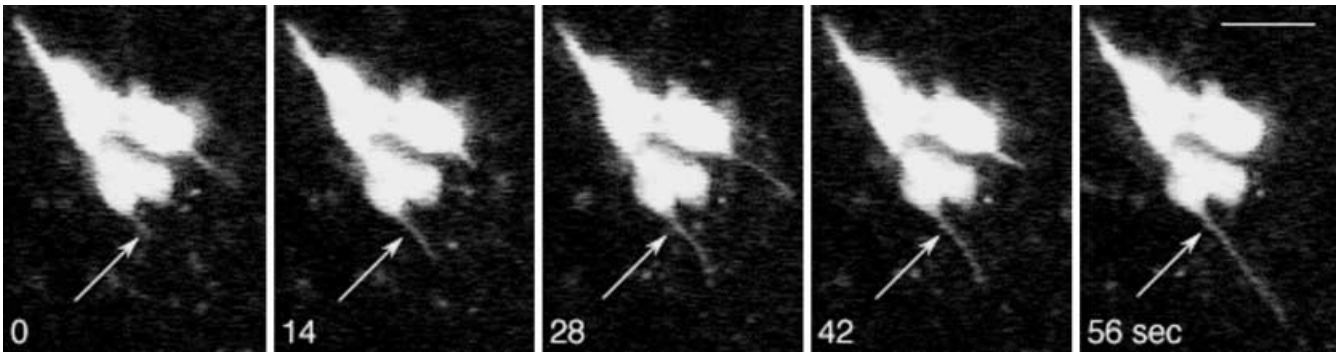

Fig. 5 The tubular character of Golgi-to-ER retrograde transport intermediates. The image shows a time-lapse sequence at 37°C of a HeLa cell expressing KDELR-GFP, a protein which constitutively cycles between the Golgi and ER. *Arrow* points to a tubule process that extends from the Golgi during this time period

Golgi membranes (Boevink et al. 1998; Nebenführ et al. 1999). Golgi stacks moved rapidly along the ER network, which is aligned along the actin cytoskeleton in plants. Treatment of cells with actin depolymerizing agents inhibited Golgi movement without causing Golgi elements to dissociate from the ER. These results suggest that Golgi units are tightly associated with the ER and use an actin-based motor to move along the ER surface. The close contact of Golgi elements to the ER in these cells presumably ensures the efficient delivery of newly synthesized cargo molecules to the Golgi apparatus, since plant cells lack the radially arranged microtubules (present in animal cells) for moving transport intermediates through the cytoplasm.

GFP-based, time-lapse studies of Golgi elements in the yeast strain *Saccharomyces cerevisiae* showed that Golgi elements, as in plant cells, are scattered and in constant motion (Wooding and Pelham 1998). Interestingly, early and late Golgi markers in these cells are not equally distributed among Golgi elements: some elements are more enriched in early, while other elements are more enriched in late markers. Exactly why this occurs is not known, but studies examining the effect of mutations that inhibit secretory traffic showed that all Golgi elements behaved similarly. Within minutes of temperature shift in sec18, sft1, and sed5 conditional mutants, the Golgi elements dispersed into vesicular structures (Wooding and Pelham 1998). This demonstrates the need for continuous membrane flow through the secretory pathway for maintenance of Golgi structure.

In mammalian cells, time-lapse imaging of the Golgi apparatus with GFP-tagged Golgi markers revealed that the overall three-dimensional arrangement of Golgi elements near the microtubule-organizing center was relatively stable over long time periods (Sciaky et al. 1997). However, there was constant formation and/or detachment of thin tubules off the rims of Golgi elements. After extending out from Golgi elements, the tubules broke off and moved at rates of 0.6 μm/s along microtubules to the cell periphery. Tubules of this type were found to label with KDELR-GFP, p58-GFP, GalTase-GFP, Cy3-shiga toxin B fragment, and Rab6-GFP (Cole et al. 1996b; Sciaky et al. 1997; White et al. 1999; Fig. 5). Because all of these Golgi proteins are capable of cycling between the Golgi and ER, the tubules are likely to represent retrograde transport intermediates returning membrane back to the ER. Treatment of cells with brefeldin A, which enhances retrograde traffic of Golgi proteins into the ER, was found to cause a dramatic proliferation of Golgi tubules carrying these and other Golgi proteins and lipids (Sciaky et al. 1997; White et al. 1999). Golgi retrograde transport intermediates are characteristically tubular in nature, and allow the Golgi to continuously exchange components with the ER.

Diffusional mobility of Golgi proteins

To understand how Golgi proteins are retained within Golgi membranes amidst constant flow of proteins through the secretory pathway, FRAP experiments have been performed to measure the diffusional mobilities of GFP-tagged Golgi membrane proteins. Results from these experiments have shown that Golgi enzymes are highly mobile in Golgi membranes with no constraints to their lateral diffusion (*D* ranging between 0.3 and 0.5 $\mu m^2/s$ and 90% of the molecules mobile; Cole et al. 1996b). These findings indicate that Golgi enzymes are not retained within Golgi membranes by being immobilized. They support, therefore, a lipid partitioning model of Golgi protein localization (Bretscher and Munro 1993). In this model, freely diffusing Golgi proteins partition into thinner regions of the Golgi bilayer and are excluded from thicker regions that are enriched in sphingolipids and sterols destined for the plasma membrane.

To determine whether vesicle transport plays any role in the rapid recovery kinetics observed in FRAP experiments of GFP-tagged Golgi enzymes, *D* and mobile fraction of the GFP chimeras was measured in ATP-depleted cells, where vesicle trafficking is inhibited (Cole et al. 1996b). There was no change in the observed diffusion parameters under these conditions, which indicated that the extensive movement of the chimeras observed within Golgi membranes by FRAP was mediated by lateral diffusion and not vesicle trafficking. The transient connec-

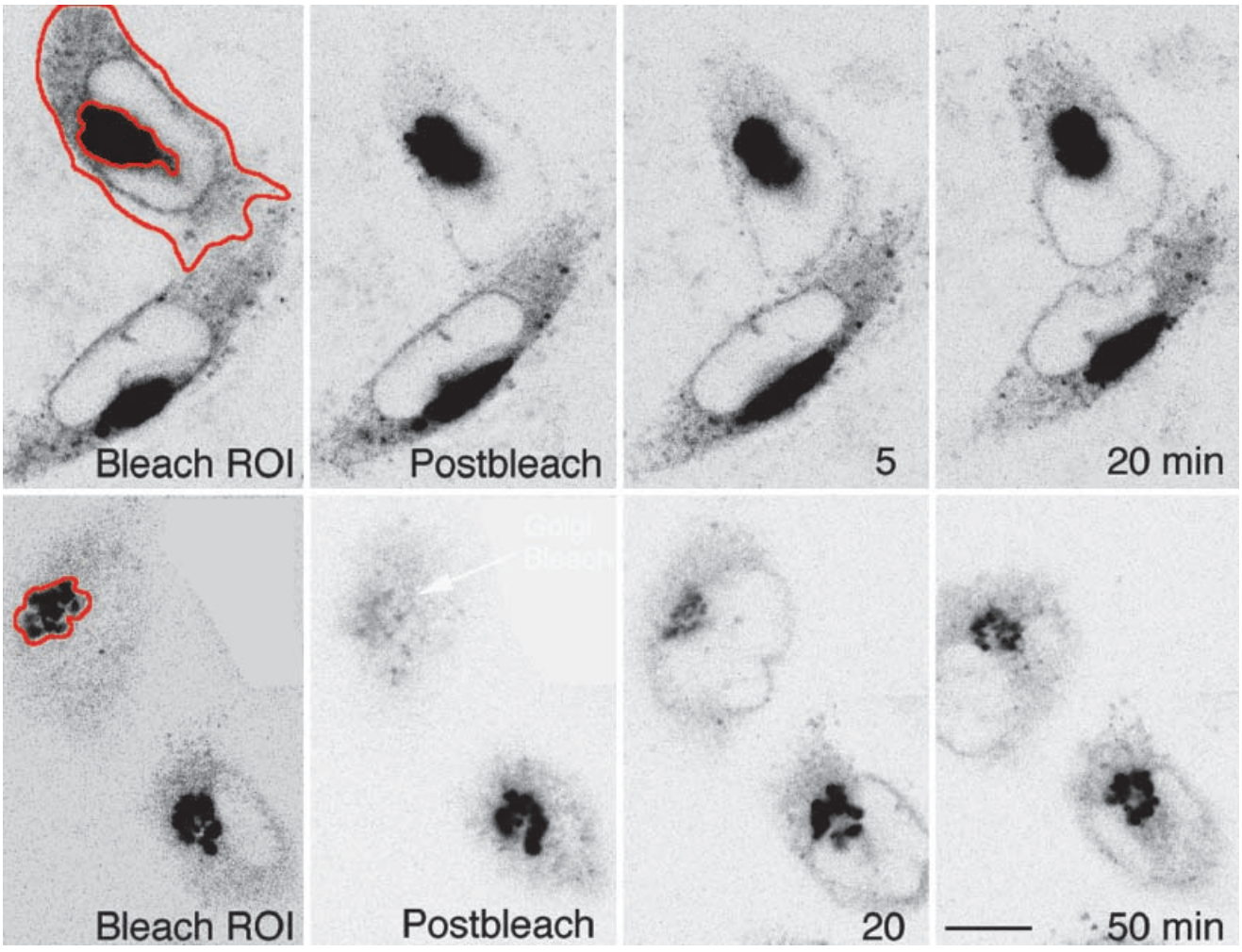

Fig. 6 Selective photobleaching of ER (*top row*) or Golgi (*bottom row*) pools of GalTase-GFP performed in cycloheximide-treated COS cells expressing the construct. The recovery of these pools over time provides evidence that GalTase constitutively cycles between the Golgi and ER (Zaal et al. 1999). *ROI* Region of interest

tions between Golgi elements by membrane tubules observed in time-lapse imaging experiments could mediate the extensive lateral exchange of Golgi resident proteins between Golgi stacks. Consistent with this, when microtubules are acutely depolymerized to prevent Golgi tubules from extending out from Golgi elements, GFP chimeras are no longer able to move efficiently between Golgi elements (Cole et al. 1996b).

Protein cycling between Golgi and ER and Golgi inheritance

The Golgi apparatus is well known to undergo dramatic structural changes when microtubules are depolymerized or when protein export from the ER is blocked. One model for explaining this dynamic behavior proposes that Golgi enzymes do not reside permanently in the Golgi apparatus, but constitutively cycle between the Golgi and ER (Cole et al. 1996a; Storrie et al. 1998; Zaal et al. 1999). Under this model, any condition inhibiting return of Golgi proteins from the ER back to the Golgi apparatus will disrupt Golgi structure, redistributing Golgi proteins to the site of inhibition. GFP-based, time-lapse imaging and photobleaching experiments have confirmed this prediction, thereby providing important support to the Golgi protein recycling model.

Golgi protein redistribution into peripheral fragments in cells whose microtubules are disrupted by nocodazole has been studied by time-lapse imaging of GFP-tagged Golgi markers (Storrie et al. 1998; Zaal et al. 1999). Upon nocodazole treatment, scattered but stationary Golgi elements were found to appear abruptly at ER exit sites, with no Golgi fragments observed tracking out from the Golgi region. Moreover, the Golgi elements arising at these sites could be loaded with secretory cargo released from the ER (Cole et al. 1996a). These data support the idea that the peripheral Golgi structures were formed by cycling of Golgi enzymes back to the ER and re-emergence at ER exit sites rather than by direct breakdown of the central Golgi. Consistent with this, Golgi fragments did not form in cells microinjected with the dominant negative mutant of Sar1, which blocks ER export. Thus, in the absence of microtubules, Golgi proteins that have recycled back to

the ER are exported into pre-Golgi intermediates that subsequently fail to translocate to the perinuclear Golgi apparatus. Maturation of these de novo structures into small Golgi stacks at peripheral ER exit sites enables the cell to re-establish secretory transport from the ER into the Golgi apparatus when microtubules are absent.

Selective photobleaching experiments have been used to determine the rate at which Golgi proteins normally cycle through the ER (Zaal et al. 1999; Dahm et al. 2001). In these experiments, Golgi or ER pools of a GFP-tagged Golgi protein were selectively photobleached in the presence of cycloheximide to inhibit new protein synthesis. Recovery into the bleached area was monitored over time to determine if the bleached and non-bleached pools of Golgi protein exchange. For GalTase-GFP, Zaal et al. (1999) found that within 20 min of selective photobleaching the ER pool of this protein ER membranes regained much of the same percentage of total cellular fluorescence they had before photobleaching (Fig. 6). A similar recovery was found when the Golgi pool of GalTase-GFP was selectively photobleached. Kinetic modeling of the data suggested that the overall time for cycling of GalTase-GFP between the ER and Golgi apparatus was about 90 min. A similar type of analysis was used to describe the cycling kinetics of VIP36-GFP (Dahm et al. 2001), a close relative of ERGIC53. The data showed that this protein resided in the Golgi for shorter periods before cycling back to the ER.

The Golgi breaks down and then reassembles during mitosis in mammalian cells. Time-lapse imaging of cells expressing GFP-tagged GalTase has shown that this occurs through a series of four discrete stages, including the appearance of peripheral Golgi fragments in prophase, total dispersal of Golgi proteins at metaphase, reappearance of peripheral Golgi fragments during telophase, and coalescence of Golgi fragments at cytokinesis (Zaal et al. 1999; Lippincott-Schwartz and Zaal 2000). A different GFP-based time-lapse imaging study reported a similar set of stages except for the stage where Golgi proteins are widely dispersed in the cytoplasm (Shima et al. 1998). The dispersed distribution of Golgi proteins in metaphase was shown by Zaal et al. (1999) to represent Golgi protein localization in the ER based on immunoelectron microscopic localization of the proteins. The fluorescent Golgi chimeras diffused within metaphase ER membranes with the same high mobility as in interphase ER membranes (Zaal et al. 1999). Future work using GFP chimeras to examine other aspects of mitotic Golgi breakdown and reformation, including Golgi fragment formation at prophase and Golgi reassembly at telophase promise to provide further insights into Golgi inheritance mechanisms.

Concluding remarks

GFP fusion proteins, used as tools to probe protein dynamics within living cells, are having a revolutionary impact on the analysis of membrane trafficking pathways and the biogenesis of secretory organelles. They have led to an understanding of some of the most fundamental properties of secretory transport, including the lifetime and kinetics of transport intermediates, the mechanisms underlying protein sorting and retention in organelles, and organelle inheritance strategies. The new information has been stimulated by developments in GFP biology, fluorescence imaging methods, as well as technical equipment. Optimized expression of GFP and the availability of GFP variants with favorable spectral properties make it easy to visualize GFP fusion proteins in living cells. Fluorescence imaging methods, such as photobleaching, no longer require custom-built systems but can be performed on user friendly, commercially available laser scanning microscopes. Cost-effective computing resources and powerful software packages, moreover, allow data to be analyzed efficiently. These developments and the range of experiments that they permit ensure that the pace of discovery in the field of membrane traffic using GFP chimeras will continue to be great.

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